Egyptian Journal of Ear, Nose, Throat and Allied Sciences (2015) **16**, 47–53

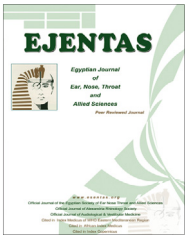
HOSTED BY

ELSEVIER

Egyptian Society of Ear, Nose, Throat and Allied Sciences

Egyptian Journal of Ear, Nose, Throat and Allied Sciences

www.ejentas.com



ORIGINAL ARTICLE

Immunohistochemical study of the possible role of osteoprotegerin OPG in inhibition of otic capsule remodeling

Yasser Shewel [a,*], Ghada Morad [b], Mokhtar Bassiouny [a], Tamer Ebaeid [a]

[a] *Department of Otolaryngology, Alexandria School of Medicine, University of Alexandria, Alexandria, Egypt*
[b] *Department of Histology, Alexandria School of Medicine, University of Alexandria, Alexandria, Egypt*

Received 13 August 2014; accepted 22 October 2014
Available online 12 November 2014

KEYWORDS

Otic capsule;
Otosclerosis;
Osteoprotegerin

Abstract *Outcome objectives:* To recognize the differential expression of OPG in the otic capsule of adult male mice compared to other selected skeletal bones both in the nearby location as the temporal bone and in distant bones as the tibia.

Methods: The present experimental study was conducted in 2011 on 20 normal adult male albino mice with an average weight of 50–60 g. Animal housing at the Physiology department (Alexandria Faculty of Medicine) followed the rules of research ethics for experimental animals approved by the Faculty of Medicine, University of Alexandria, Egypt. The following bone specimens were harvested from normal adult male albino mice by microdissection: (1) temporal bone, (2) otic capsule bone surrounding the cochlea, and (3) tibia and stained immunohistochemically for anti-OPG monoclonal antibody. Positive staining was graded and analyzed using image softwares that measure the staining intensity as units of pixels/microscopic field examined at 400 magnification.

Results: OPG was detected as a brown DAB chromogen staining of tissue components expressing a positive OPG monoclonal antibody immune reactivity.

Statistical analysis of the results revealed that high OPG level concentrations were found in the otic capsule followed by the temporal bone and finally the tibia.

Conclusion: The findings highlight the role of OPG in inhibition of otic capsule remodeling.

1. Introduction

Unlike all other bones in the human skeleton, the otic capsule undergoes very little remodeling after development, possibly due to local factors produced by the inner ear.[1–3]

Different types of bone cells normally integrate to achieve a balanced state of bone metabolism adapting the otic capsule for the auditory and equilibrium functions occurring within

* Corresponding author at: ENT Department, Alexandria School of Medicine, University of Alexandria, Ramleh Station, Alexandria, Egypt. Tel.: +20 1220149937.
E-mail address: yshwel@yahoo.com (Y. Shewel).
Peer review under responsibility of Egyptian Society of Ear, Nose, Throat and Allied Sciences.

http://dx.doi.org/10.1016/j.ejenta.2014.10.006

it. For instance, osteoblasts are responsible for bone deposition; whereas osteoclasts lead the process of bone remodeling through its bone resorption activity.[4]

By the beginning of the last decade, osteoprotegerin (OPG) was found to act as a key regulator of bone metabolism. OPG is a naturally occurring protein with potent osteoclastogenesis inhibitory activity, which plays a key role in the physiological regulation of osteoclastic bone resorption. OPG is produced by osteoblasts and marrow stromal cells.[5] OPG inhibits differentiation, survival, and fusion of orthoclastic precursor cells and suppresses activation and promotes apoptosis of osteoclasts. Thus, under expression of OPG would show severe osteoporosis, whereas over expression of OPG would result in osteopetrosis.[6]

The interest for this research is driven by the concern of otologists to explain the pathogenesis of otosclerosis as one of the most challenging diseases of the otic capsule.

Otosclerosis is a localized disease of bone remodeling within the otic capsule of the temporal bone. Unlike other similar bone diseases, it does not occur outside of the temporal bone. These lesions seem to begin by bypassing normal inhibition of otic capsule remodeling; consequently resorption of stable otic capsule bone occurs, followed by a reparative phase with bone deposition.[7]

Otosclerosis is among the most common causes of acquired hearing loss. It is considered as a multifactorial disease, caused by both genetic and environmental factors. Despite the extensive research, the process of development of otosclerosis remains unclear.[8]

The present study aimed at characterizing osteoprotegerin (OPG) as one of the molecules responsible for the unique pattern of bone remodeling in the otic capsule.

Immunohistochemical demonstration of OPG within the otic capsule of adult normal mice might throw light on the correlation between the density and distribution of OPG and the development of otosclerotic foci.

In order to confirm this hypothesis, the expression of OPG in the otic capsule of mice has been compared to its expression in other skeletal bones known for undergoing a relatively high rate of turnover and remodeling, namely the surrounding temporal bone and the tibia.

2. Material

2.1. Experimental animals

The present experimental study was conducted in 2011 on 20 normal adult male albino mice with an average weight of 50–60 g. Animal housing at the Physiology department (Alexandria Faculty of Medicine) followed the rules of research ethics for experimental animals approved by the Faculty of Medicine, University of Alexandria.

2.2. Immunohistochemical kit for osteoprotegerin

Osteoprotegerin is a biotinylated monoclonal anti-mouse antibody (OPG/TNFRSF11B). The kit is a product by Quantikine and was purchased via the Egyptian agency for R&D Systems. It was provided in a 50 μg vial containing 50 μg of bovine serum albumin per 1 μg antibody lyophilized in a 0.2 μm filtered solution of phosphate buffered saline.

3. Methods

3.1. Harvesting of the specimens

The following specimens were collected from normal adult male albino mice by microdissection: (1) temporal bone (2) otic capsule bone surrounding the cochlea, and (3) tibia.

3.2. Preparation and processing

The bone samples (temporal bone, otic capsule and tibia) were decalcified in 3% trichloroacetic acid solutions at room temperature for 14–21 days. When adequately softened, the samples were further processed into paraffin blocks 5–6 μ thick sections were mounted on ordinary and on positively charged glass sections for preparation of hematoxylin and eosin sections and OPG immunolabeled sections respectively.

3.3. Histological technique

Histological technique included light microscopic examination of paraffin sections using routine hematoxylin and eosin (H&E) stain.

3.4. Immunohistochemical technique

Sections were stained with the universal polyclonal kit (Thermo scientific). Antigen retrieval was performed (boiling slides in 2% citrate buffer for 30 min). Blocking of endogenous peroxidase activity was performed by incubation with 0.6% H_2O_2. Nonspecific protein staining was blocked by incubation with the blocking agent provided. Subsequently, sections were incubated with the primary antibody (at a concentration of 5 and 10 mg/ml (i.e. 1:5 and 1:10 dilution) overnight at 40 °C. On day 2: The secondary antibody was applied for 30 min at room temperature followed by the linking agent for another 30 min. Diaminobenzidine (DAB) was used as the chromogen. Each two steps were separated by 2× wash in Tri-HCl buffer (Lab Vision Co., Fremont, CA, USA). Positive staining was graded and analyzed using image software that measures the staining intensity as units of pixels/microscopic field examined at 400 magnification.[9]

3.5. Method for image analysis

The concept for applying image analysis software to the OPG immunolabeled sections of the otic capsule, the associated part of the petrous temporal bone and the tibia was to quantitatively assess and compare the expression of the OPG within the tissue components of these bones. The sites expressing a positive immune reaction with the OPG monoclonal antibody are identified as brown deposits of the DAB chromogen. The stronger the immune reaction, the darker the DAB chromogen intensity. The calculation of the DAB chromogen density was done by applying digital image analysis. Digital images of stained sections from the 20 experimented animals were examined with a computer-assisted light microscope (Olympus microscope – equipped with Spot 16-bit digital camera (1280×1024 pixel)). Images were viewed and recorded using computer program MAtLAB software version 5.5 (Image J,

the mathworks, Inc., USA). The maximum, minimum and integrity of intensity color was based on Gray-level acquisition, analysis of the data was carried out by reading 10 fixed areas in one image. The records were taken for 5 images.

3.6. Interpretation of image analysis results

The mean values of each reaction were based on the mean of the pixel number for each examined microscopic area. Interpretation of the results for pixel analysis of OPG immunostaining was built upon the software program for image analysis which expressed OPG staining density by a value that was inversely proportional to the measured pixel number. Accordingly, the larger the pixel number the weaker the OPG immune expression.[10]

3.7. Statistical analysis

Data were fed to the computer using IBM SPSS software package version 20.0. Quantitative data were described using mean, standard deviation, median, minimum and maximum. Comparison between different groups was analyzed using the *F*-test (ANOVA) and the Post Hoc test (LSD) for pair wise comparison. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

4. Results

4.1. Histological results

The H&E stained histological sections of the petrous part of the temporal bone demonstrated the normal microscopic anatomy of hemisections passing across the mice otic capsule. The H&E section of the right tibia demonstrated the normal architecture of the epiphysis, metaphysis and diaphysis.

4.2. Immunohistochemical results

OPG was detected as a brown DAB chromogen staining of tissue components expressing a positive OPG monoclonal antibody immune reactivity. The reaction appeared as brown deposits in cells but was rather a homogenous brownish tinge in fluids and tissue ground substance.

During the light microscopic examination of immunohistological sections of the dissected mice sections, the positive OPG immune reaction could be localized in cells as the osteocytes and osteoblasts of bony tissue of the otic capsule, the adjacent petrous part of the temporal bone and the tibia. It could be detected to a lesser extent in chondrocytes within the hyaline cartilage forming the epiphyseal plate of the tibia and in the epithelial cells lining the membranous labyrinths of the semicircular canals.

The intensity of the OPG immune expression in the boney labyrinths of the otic capsule was generally detectable at the level of the basal portion as well as near the apical part (Fig. 1). On the other hand, several other microanatomical structures within the otic capsule lacked the tendency for OPG immune expression for instance, the stria vascularis bouldering the lateral wall of the scala media within the cochlea, in which no OPG immune reactivity was detectable (Fig. 2).

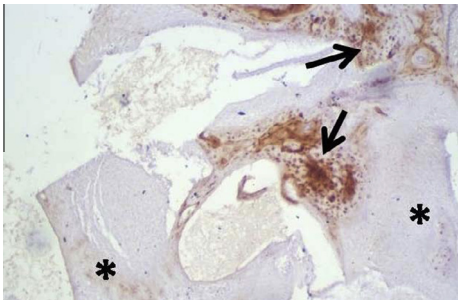


Figure 1 Light photomicrograph for a cross section proximal to the apex of the otic capsule and the adjacent petrous part of temporal bone (T). The boney specules of the otic capsule demonstrate evident strong positive OPG immune reaction (↑) compared to the faint non specific reaction depicted in the petrous temporal bone (*).

In addition, to the variation in the intensity of OPG immune reaction among the bone trabeculae of the otic capsule, it was observed that similar differences were applicable for the endolymph and perilymph filling the scala media and scala tympani respectively. The endolymph exhibited microscopically non detectable OPG immune staining. On the other hand, the perilymph in the scala tympani and other cavities within the otic capsule reacted positively to OPG monoclonal antibody immune staining. The pattern of the reaction was rather a homogenous, non specific brown tinge compared to the specific strong positive reaction observed in osteocytes within the adjacent bone trabeculae (Figs. 2 and 3).

Regarding the temporal bone, it was noted that the OPG expression was generally less evident compared to the bone trabeculae of the otic capsule. Sections passing across the petrous part of the temporal bone away from the otic capsule revealed faint non specific OPG immune reactivity. However, a detectable OPG positive immune staining was observed in sections in the temporal bone passing nearer to the otic capsule. The reaction appeared as a localized weak brownish tinge in the ground substance between the air cells (Fig. 4).

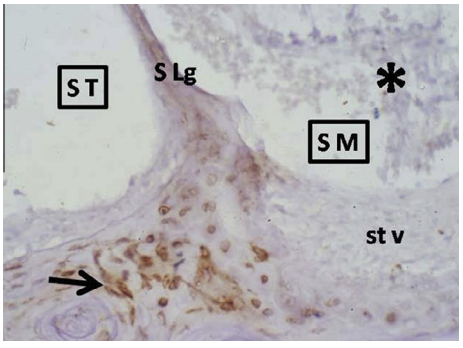


Figure 2 High power view of a section across the mouse right otic capsule passing through the scala media (SM), the spiral ligament (SLg), and the scala tympani (ST). The lateral wall of the otic capsule is formed of compact bone revealing many OPG positive osteocytes (↑) compared to the negative immunostaining of the stria vascularis (st v) and the endolymph (∗) filling the scala media.

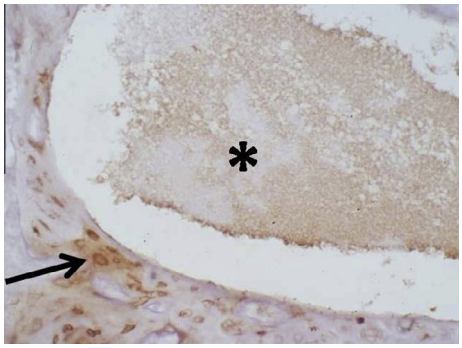


Figure 3 High power view of a cross section of the scala tympani within the mouse right otic capsule. The cavity of the scala tympani is filled with residual perilymph showing faint homogenous brownish tinge (*). Note the specific positive OPG immunostaining of the osteocytes (↑) within the layer of compact bone forming the wall of the cavity.

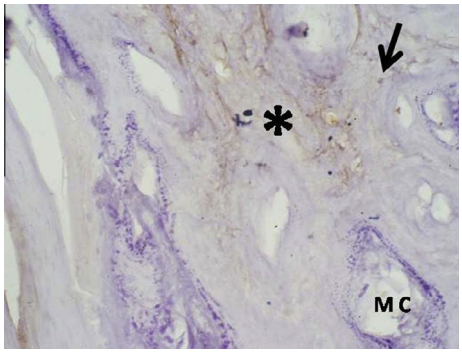


Figure 4 Light photomicrograph for OPG immunostaining of a normal rat right petrous part of temporal bone. The boney ground substance (*) between the mastoid air cells (MC) shows weak brown deposits. Note that few scattered bone cells (↑) show a specific OPG positive immune reaction.

The compact bone forming the outer tables of the shaft and the trabeculae of cancellous bone at the metaphysis of the tibia demonstrated an overall faint affinity to express OPG immune reaction. Compared to the intensity of OPG expression in the otic capsule, the reaction is apparently poor (Fig. 5).

4.3. Statistical results for image analysis of the intensity for (OPG) immunostaining

Interpretation of the results for pixel analysis of the OPG immunostaining was built upon the software program for image analysis which expressed the OPG staining density by a value that was inversely proportional to the measured pixel number. Accordingly, the larger the pixel number the weaker the OPG immune expression. The description for strong, moderate and weak OPG immune expression was founded upon the quantitative grading of the mean pixel value into a scale for values below 100, a scale ranging between 100 and 140 and a scale above 140 respectively (Table 1).

For the *otic capsule near its basal part*, OPG immunoexpression was the strongest in 10% of the experimented mice

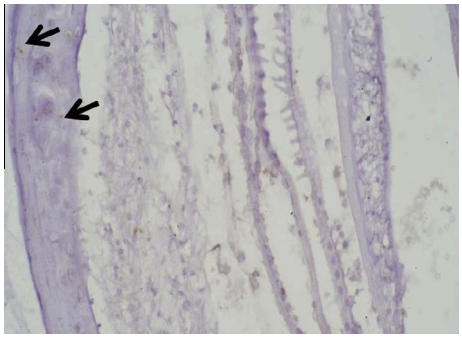


Figure 5 Light photomicrograph of normal mouse tibia demonstrating an overall faint OPG immunohistochemical reaction. A brownish positive immunostaining is depicted in scattered bone cells (↑) within the outer table of the compact bone.

(mean pixel value 89.32 and 96.71). It was moderate (mean pixel value ranging between 107 and 138) in 80% of the mice. A weak OPG expression was depicted in 10% of the mice (mean pixel values 142.78 and 172.20).

The *apical part of the otic capsule* revealed statistical results for the OPG mean pixel values approximating those of the basal part of the otic capsule. A strong OPG immunostaining was expressed in 15% of mice (mean pixel values 95.82.93, 89.32, 95.82), while 20% showed a weak OPG expression (mean pixel values 145.13, 145.13, 150.09, 150.59). More than half of the experimented mice sample (65%) demonstrated a moderately positive OSP labeling (mean pixel value ranging between 106 and 131).

On analyzing the mean pixel values reflecting the OPG immune reaction in *the temporal bone*, it was obvious that all mice failed to express a strong OPG labeling (0% for the mean pixel value below 100). Only 15% of the mice demonstrated a moderate OPG immune reaction (mean pixel values 123.99, 123.99, 140.50). The remaining 85% of the mice sample expressed a weak OPG immune reactivity (mean pixel values ranging between 142 and 170). (Table 1)

Regarding the OPG immune expression in *the perilymph* within the otic capsule, a strong reaction could be depicted in 15% of the mice (mean pixel values 93.33, 99.16, 99.16). A moderate OPG reaction could be demonstrated in 35% of the experimented rat sample (mean pixel values ranging between 114 and 168); whereas 50% of the animals expressed a weak reaction (mean pixel values ranging between 143.52 and 160.48).

Compared to the otic capsule, perilymph and the surrounding temporal bone, all of the experimented mice failed to reveal neither strong nor moderate OPG immune expression *for the tibia* (0% for mean pixel values below 100 and 0% for values between 100 and 140). 100% of the experimented rats gave weak positive OPG immune labeling (mean pixel values ranging between 141 and 186).

The statistical comparison between the mean pixel values for OPG immune reactivity in the different investigated parameters indicated that the highest values were demonstrated in the tibia, followed by the temporal bone, the perilymph, and in the base of the otic capsule and was the lowest in the apical part of the otic capsule. By interpreting these values in correlation to the corresponding strength of the OPG expression, this indicated that the greatest strength

Table 1 Quantitative grading of the expressed OPG–Immune.

Percentage of mice across investigated parameters	Strong OPG Immune–expression (mean pixel value below 100) (%)	Moderate OPG Immune–expression (mean pixel value ranging between 100 and 140) (%)	Weak OPG Immune–expression (mean pixel value above 140) (%)
Basal part of the otic capsule	10	80	10
Apical part of the otic capsule	15	65	20
Temporal bone	0	15	85
Perilymph	15	35	50
Tibia	0	0	100

Table 2 Mean, standard deviation and median values of number of pixels measuring the density of immunostaining for (*N.B:* the OPG staining density was expressed by a value that was inversely proportional to the measured pixel number. Accordingly, the larger the pixel number the weaker the OPG immune expression).

	OSP-base otic capsule	OSP-apex otic capsule	OSP-temporal bone	OSP-perilymph	OSP-tibia
Min.–Max.	96.71–176.30	89.32–150.59	123.99–170.90	93.33–168.61	138.46–189.09
Mean ± SD	126.11 ± 17.80	123.33 ± 17.48	148.59 ± 12.77	137.70 ± 22.74	162.52 ± 13.79
Median	117.72	121.85	148.21	141.06	158.69
p			$<0.001^*$		
p_1		0.611	$<0.001^*$	0.037^*	$<0.001^*$
p_2			$<0.001^*$	0.010^*	$<0.001^*$
p_3				0.049^*	0.012^*
p_4				$<0.001^*$	

p: p value for F test (ANOVA).
p_1: p value for Post Hoc test (LSD) for comparing between OPG-base otic capsule and each other place.
p_2: p value for Post Hoc test (LSD) for comparing between OPG-apex otic capsule with OSP-temporal bone, OPG-perilymph and OSP-tibia.
p_3: p value for Post Hoc test (LSD) for comparing between OPG-temporal bone with OSP-perilymph and OPG-tibia.
p_4: p value for Post Hoc test (LSD) for comparing between OPG-perilymph and OPG-tibia.
* Statistically significant at $p \leqslant 0.05$.

could be demonstrated in the base and apex of the otic capsule, followed by perilymph. It was the least in the temporal bone and in the tibia (Table 2).

5. Discussion

The pathogenesis of otosclerosis has been a mystery for centuries. Otosclerosis is characterized by abnormal focal bone remodeling in the bony otic capsule or in rare cases, the ear ossicles.

Because of recent advances in bone research, it is now known that RANK, RANKL and OPG are the primary regulators of bone metabolism and that the balance between these three molecular factors is fundamental in determining the kinetics of bone turnover at the local level.[11]

OPG is a powerful inhibitor of bone remodeling. OPG knockout mice show severe osteoporosis,[12] whereas overexpression of OPG in transgenic mice causes osteopetrosis.[13]

The otic capsule, which is the hardest bone in the body, is unique in its morphology and development. After the onset of ossification at gestational week 16 in humans, the otic capsule forms a unique functional unit in which growth, modeling, and remodeling in bone are virtually absent.[14]

This lack of remodeling of bone is the basis for non healing of fractures of the temporal bone. However, remodeling of bone within the otic capsule does occur in certain pathologic disorders such as otosclerosis and Paget's disease.

The biologic mechanism that controls bone metabolism in the otic capsule and that may be responsible for its low rate of bone turnover within the otic capsule remains largely unknown at the present time.

The present study targeted the localization of OPG in the otic capsule surrounding the cochlea, the temporal bone and the tibia of normal adult mice. The target for the research done aimed at exploring the answer to the frequently addressed question about "why otosclerosis is unique for the otic capsule?"

One of the challenges during processing of the dissected cochlea and tibia was to reach out for the optimum fixation and decalcification protocol that would realize three main purposes:

1. Bone softening thus permitting adequate sectioning and staining of bone.
2. Preservation of bone architecture and bone cell morphology for microscopic examination.
3. Preservation of tissue antigenicity to serve the purpose of immunohistochemical identification of OPG in bone cells.

Practically, immunohistochemical investigation of paraffin-embedded bone tissue is often hampered by fixation and decalcification procedures. Routine bone decalcifying agents such as EDTA and nitric acid not only destroy tissue antigenicity by denaturing of cell specific antigens but also entail the

application of time consuming procedures.[15] Therefore, we reverted to 3% trichloroacetic acid as a gentle, less drastic chelating agent that achieves optimum bone softening within 3 weeks without denaturation of tissue protein and without inducing architectural distortion.[16]

The software program applied to analyze the pixel values indicating the immune-staining intensities, provided a rough quantitative estimate for the density of OPG in the anatomical compartments of the inner ear based upon microscopic immunohistochemical data.

Different concentrations of OPG in five different places in experimental animals namely the base of otic capsule, apex of otic capsule, the temporal bone, the perilymph of the inner ear and the tibia were measured using immunohistochemistry and image analysis as methods.

Results found that high OPG level concentrations were found in the base and apex of the otic capsule followed by the perilymph then temporal bone and finally the tibia, which mean that the higher concentration was in the otic capsule and the lowest was found in the tibia, and differences in concentrations were statistically different between all areas.

And this explains the much lower rates of bone remodeling in the otic capsule secondary to the high level of OPG concentration there and the high rate of bone turnover in the tibia secondary to the low level concentration of OPG in the tibia.

When applied to the otic capsule, normal OPG levels inhibit remodeling and therefore protect against otosclerosis. External causes disturbing the OPG homeostasis in the otic capsule would subsequently enhance otosclerosis.[17]

Several experimental and clinical studies focused on the correlation between the rate of turnover in the otic capsule, OPG expression and the localization of otosclerotic foci.

Zehnder et al. and Andreas found that active remodeling process in the OPG knockout mice has many similarities to otosclerosis seen in human temporal bones. And they get to the conclusion that the histopathological and pathophysiological findings in OPG knockout mice support the hypothesis that OPG is important in the inhibition of bone remodeling within the otic capsule and the maintenance of normal auditory function.[18]

Zehnder et al. compared the relative amount of OPG mRNA that was present in the cochlear soft tissue, otic capsule bone, calvilear bone, and cortical bone of the femur and was compared with the mRNA levels for RANKL in those same tissues. The results found that OPG and RANKL were expressed in the femur in roughly a 1:1 ratio. However, the bone of the otic capsule surrounding the cochlea showed an increased ratio of OPG and RANKL. This study got a conclusion that the ratio of OPG to RANKL is critical in determining the kinetics of local bone remodeling.[19]

Furthermore, Kristiansen suggests that OPG is produced in high levels in the cochlea, and it diffuses into the surrounding otic capsule by way of a lacuno-canalicular system.[19]

The correlation between the histological and the immunohistochemical results allowed tracking of the distribution of OPG expression in the different anatomical compartments of the otic capsule. OPG could be localized, with variable immunoexpression densities, within the bony specules forming the lateral walls of the base and apex of the cochlear, in the modulus, in the perilymph filling the scalae vestibuli and tympani as well as in the endolymph filling the scala media.

The results coincide with the findings obtained by Kanzaki et al. and Bloch et al. , who studied the location of OPG in the inner ear and the temporal bone in relation to otosclerotic foci.[20,21] However, in contrast to these studies, the present work was more concerned about the exact localization of OPG in relation to the components of the inner ear bony labyrinths, both at the cellular as well as at the anatomical levels, rather than stating its overall positive immunoreactivity in the otic capsule.

Since OPG has been proved to be protective against bone remodeling,[19] odontologists used it as an antiresorption drug that would promote bone regeneration and inhibit resorption, thereby improving the clinical outcome for distraction osteogenesis.[22] In addition to its applications in odontology, OPG has been tried for treatment of bone cancer, myeloid myelomas and other osteolytic bone disorders to inhibit excessive bone resorption and reduce bone fractures in these patients.[23]

6. Conclusion and recommendation

Statistical analysis of the results revealed strong OPG expression in the apex, base and perilymph inside the cochlea. The lack of OPG in the temporal bone and in the tibia confirms the peculiar structural and metabolic status of the otic capsule thus confirming the possible role of a disturbed OPG signaling as a potent mechanism precipitating the development of otosclerosis.

Conflict of interest

None declared.

References

1. Thys M, Van Camp G. Genetics of otosclerosis. *Otol Neurotol*. 2009;30(8):1021–1032.
2. Zehnder AF, Kristiansen AG, Adams JC, Kujawa SG, Merchant MJ, McKenna MJ. Osteoprotegrin knockout mice demonstrate abnormal remodeling of the otic capsule and progressive hearing loss. *Laryngoscope*. 2006;116(2):201–206.
3. Stankovic KM, McKenna MJ. Current research in otosclerosis. *Curr Opin Otolaryngol Head Neck Surg*. 2006;14(5):347–351.
4. Sims NA, Gooi JH. Bone remodeling: multiple cellular interactions required for coupling of bone formation and resorption. *Semin Cell Dev Biol*. 2008;19(5):444–451.
5. Kostenuik PJ, Shalhoub V. Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. *Curr Pharm Des*. 2001;7(8):613–635.
6. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys*. 2008;473(2):139–146.
7. Chole RA, McKenna M. Pathophysiology of otosclerosis. *Otol Neurotol*. 2001;22(2):249–257.
8. Markou K, Goudakos J. An overview of the etiology of otosclerosis. *Eur. Arch. Otorhinolaryngol.*. 2009;266(1):25–35.
9. Shi SR, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. *J Histochem Cytochem*. 2007;55(2):105–109.
10. Collins TJ. Image J for microscopy. *Biotechniques*. 2007;43:25–30.
11. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol Med*. 2001;79(5–6):243–253.

12. Udagawa N, Takahashi N, Yasuda H, et al. Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Endocrinology*. 2000;141(9):3478–3484.
13. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell*. 1997;89(2):309–319.
14. Sorensen MS, Frisch T, Bretlau P. Dynamic bone studies of the labyrinthine capsule in relation to otosclerosis. *Adv Otorhinolaryngol*. 2007;65:53–58.
15. Mullink H, Henzen-Logmans SC, Tadema TM, Mol JJ, Meijer CJ. Influence of fixation and decalcification on the immunohistochemical staining of cell-specific markers in paraffin-embedded human bone biopsies. *J Histochem Cytochem*. 1985;33(11):1103–1109.
16. Neves Jdos S, Omar NF, Narvaes EA, Gomes JR, Novaes PD. Influence of different decalcifying agents on EGF and EGFR immunostaining. Acta Histochem. 2011;113 (4):484–488
17. Bloch SL, Sorensen MS. Three-dimensional reconstruction of the otosclerotic focus. *Acta Otolaryngol*. 2010;130(4):429–434.
18. Zehnder AF, Kristiansen AG, Adams JC, Kujawa SG, Merchant MJ, McKenna MJ. Osteoprotegrin knockout mice demonstrate abnormal remodeling of the otic capsule and progressive hearing loss. *Laryngoscope*. 2006;116(2):201–206.
19. Zehnder AF, Kristiansen AG, Adams JC, Merchant SN, McKenna MJ. Osteoprotegerin in the inner ear may inhibit bone remodeling in the otic capsule. *Laryngoscope*. 2005;115(1):172–177.
20. Kanzaki S, Takada Y, Ogawa K, Matsuo K. Bisphosphonate therapy ameliorates hearing loss in mice lacking osteoprotegerin. *J Bone Miner Res*. 2009;24(1):43–49.
21. Bloch SL, Kristensen SL, Sorensen MS. The viability of perilabyrinthine osteocytes: a quantitative study using bulk-stained undecalcified human temporal bones. *Anat Rec (Hoboken)*. 2012;295(7):1101–1108.
22. Wycherly BJ, Berkowitz F, Noone AM, Kim HJ. Computed tomography and otosclerosis: a practical method to correlate the sites affected to hearing loss. *Ann Otol Rhinol Laryngol*. 2010;119(12):789–794.
23. Andelkovic Z, Katic V, Mihajlovic D, Zivkovic V, Petrovic A, Lalosevic D. Osteoprotegerin – a neutralizing receptor, protector of bones and a potential antiresorptive agent. *Med Pregl*. 2005;58(7–8):362–367.